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Antitumor and antimetastatic activities of rhamnogalacturonan-II-type polysaccharide isolated from mature leaves of green tea *via* activation of macrophages and natural killer cells

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ABSTRACT

To investigate the antitumor and antimetastatic polysaccharide from the mature leaves of green tea, GTE-II was purified using size exclusion chromatography. GTE-II consisted of 15 different sugars including rarely observed sugars such as 2-O-methyl-fucose, 2-O-methyl-xylose, apiose, aceric acid, 3-deoxy-D-*manno*-2-octulosonic acid, and 3-deoxy-D-*lyxo*-2-heptulosaric acid, which were characteristics of pectic polysaccharide rhamnogalacturonan-II. Treatment of peritoneal macrophages with GTE-II not only increased interleukin (IL)-6 and IL-12 production, but also had significantly increased tumoricidal activity against Yac-1 tumor cells than those obtained from untreated mice. In an assay of natural killer (NK) cell activity, intravenous administration of GTE-II significantly stimulated NK cytotoxicity against Yac-1 tumor cells. Furthermore, the depletion of NK cells by injection of rabbit anti-asialo GM1 serum eliminated the inhibitory effect of GTE-II on B16BL6 melanoma cells. These data suggest that GTE-II inhibits tumor metastasis, and its antitumor effect is associated with activation of macrophages and NK cells.

1. Introduction

For the past few decades, cancer incidence has increased markedly, and it is the cause of the highest mortality rate in most parts of the world. When cancer cells start to invade, metastasis occurs at the primary tumor site by degradation of the basement membrane and the extracellular matrix, and their penetration into the vascular or lymphatic circulation [1]. Dissemination of tumor cells has become a big problem because the majority of cancer-related mortality is not due to the primary tumor itself but metastasis of tumor cells to secondary sites through a series of events collectively such as the metastatic cascades in patients with various types of cancers [2–4]. Therefore, inhibiting cancer metastasis is considered as a crucial goal in cancer therapy. Currently, cancer treatments include surgery, chemotherapy, and radiation therapy; however, their side effects such as attacking cancer cells as well as normal cells, have been reported.

In recent years, botanical polysaccharides from food or herbal medicines such as mushroom, algae, lichens, and plants have received much attention as an alternative to traditional cancer medicines, because they have a broad spectrum of therapeutic properties and relatively low toxicity for normal cells [5]. Polysaccharides can act as immunomodulators and stimulate the immune system, and this eventually contributes to elimination of tumors and foreign invaders [6]. Polysaccharides modulate immune function in many ways such as promoting the proliferation and differentiation of lymphocytes, and secretion of various lymphokines [7]. Activation of the innate immune system plays an important role in the defense against foreign antigens and malignant self-cells such as tumors [8]. In innate immunity, macrophages and dendritic cells are important because they perform the first line of defense against potential pathogens [9]. When pathogens penetrate an epithelial barrier, they are engulfed and digested by macrophages *via* lysosomal enzymes [10]. NK cells, another

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major component of the innate immune system, have strong cytolytic activity against tumors and virus-infected cells. In addition, macrophages can activate NK cells to attack tumors, and thus, cooperation between macrophages and NK cells may play a major role in the defense against tumors and pathogens [11].

Green tea (*Camellia sinensis* L.) is one of the most popular beverages consumed worldwide in its green, black, or oroolong form, and has become an important agricultural product [12]. It contains many compounds such as polyphenols, polysaccharides, and vitamins [13]. In particular, epigallocatechin gallate (EGCG), a major polyphenolic compound in tea extracts, has a variety of properties such as antioxidant activity [14], anti-carcinogenic properties [15], and reduces the risk of cardiovascular diseases [16]. Numerous data, including those from the enumerated studies, have indicated that the biological and pharmacological effects of tea are specific to EGCG in immature tea leaves. However, EGCGs cannot account for all of the observed clinical effects of tea. There is still the possibility that other biologically active ingredients such as specific polysaccharides may exist in mature tea leaves. Therefore, in order to discover new physiologically active polysaccharides from mature tea leaves, we isolated polysaccharides from pectinase digests of mature tea leaves and elucidate the mechanisms by which they stimulate the immune system.

2. Materials and methods

2.1. Chemicals

The green tea used was cultivated in Hadong, Gyeongnam, Korea, and the leaves harvested in 2009. Pectinase from *Aspergillus niger* was purchased from Sigma (St. Louis, MO, USA) and poly (1,4-α-D-galacturonide) glycohydrolase (endo-PGase, EC 3.2.1.15) was purified from pectinase (Sigma) by the procedure of Thibault and Mercier. Sephadex G-100 was obtained from Pharmacia (Uppsala, Sweden).

2.2. Isolation and purification of polysaccharide from the leaves of green tea

The leaves of green tea (1 kg) were sliced and decolorized sequentially by ethanol, methanol:chloroform (1:1), and acetone. Resulting precipitates were treated with pectinase at a final concentration of 0.5%. The enzymatic hydrolysates were centrifuged at 6000 rpm for 20 min to remove residue, then 4 times the volume (v/v) 95% ethanol was added, and stirred for 24 h to precipitate polysaccharides. The resulting precipitates were dissolved in a small amount of distilled water, followed by dialysis using a dialysis tube (MW cut-off 12,000) for 2 days, and crude polysaccharide (GTE-0) was obtained after lyophilizing. GTE-0 was purified by one column chromatography (4 × 120 cm) on Sephadex G-100 equilibrated with 50 mM ammonium formate buffer (pH 5.5). Finally, three major purified polysaccharides (GTE-I, GTE-II, and GTE-III) with different molecular weights were obtained and lyophilized after desalting by dialysis.

2.3. General analytical methods

Total carbohydrate, uronic acid, and protein were determined using phenol-H_2SO_4 [17], *m*-hydroxybiphenyl [18], and Bradford's method [19] with Bio-Rad (Hercules, CA, USA) reagents, using galactose, galacturonic acid, and bovine serum albumin as the respective standards. The amount of 2-keto-3-deoxy-D-*lyxo*-heptulosaric acid (Dha) and 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo) were colorimetrically determined by the modified thiobarbituric acid (TBA) method [20]. The sugar components of polysaccharides were analyzed using alditol acetate method after hydrolysis of polysaccharides with 2 M trifluoroacetic acid (TFA) for 1.5 h at 121 °C [21], and analyzed *via* gas chromatography (GC) using the procedure of Zhao et al. [22]. Kdo and Dha were analyzed by GC as alditol acetates according to the modified methods of York et al. [23] and Stevenson et al. [24]. Briefly, the samples were partially hydrolyzed under mild acid conditions (0.1 M TFA, 100 °C, 1 h), and the resulting hydrolyzates were reduced with sodium tetradeutero-borate ($NaBD_4$). The samples were then treated with 2 M TFA for 1 h at 121 °C, and the resulting lactones were reduced with $NaBD_4$ under neutral conditions. The products were further treated with 2 M TFA and $NaBD_4$ to reduce Dha completely. After acetylation, the resulting carboxyl reduced alditol acetates were analyzed through GC (6000 series GC; Young-Lin Co., Anyang, Korea) using an SP-2380 capillary column (0.2-μm film thickness, 0.25 mm i.d. × 30 m; Supelco, Bellefonte, PA, USA). The molar percentage was calculated from the peak areas and response factors using a flame-ionization detector (FID). High-performance size-exclusion chromatography (HPSEC) was performed on an HPLC-1260 Infinity instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with a column of Superdex 75 GL (GE Healthcare Bio-Sciences, Uppsala, Sweden). Ten microliters of each polysaccharide solution (10 mg/mL) was analyzed by using an isocratic mobile phase (50 mM formate buffer, pH 5.0) at a flow rate of 0.5 mL/min and room temperature. Molecular weights of polysaccharides were estimated from the calibration curve constructed for standard pullulans (P-200, 100, 50, 20, 10, and 5; Showa Denko Co. Ltd., Tokyo, Japan).

2.4. Methylation analysis

Methylation analysis was performed according to a slightly modified Hakomori method [25]. In brief, 0.5–1 mg of samples was dissolved in 1 mL of dimethyl sulfoxide (DMSO) and the methylsulfinyl carbanion was added repeatedly until the poly- and oligo-saccharides were completely converted into polyalkoxides, as determined by triphenylmethane. For methylation of samples containing 2-O-methyl-fucose (2-Me-Fuc) and/or 2-O-methyl-xylose (2-Me-Xyl), trideutriomethyl iodide (CD_3I) instead of methyl iodide (CH_3I) was used for distinguishing from methyl group-containing monosaccharides [26]. Oligosaccharides containing Kdo or Dha residues were pre-reduced with $NaBD_4$ and permethylated as described elsewhere [23]. Meanwhile to obtain the fully methylated products from the polysaccharide sample, repeated (double or triple) methylation processes were carried out. The methylated poly- and oligo-saccharides were recovered using a Sep-Pak C_{18} cartridge. Each methylated polysaccharide was hydrolyzed with 1 M TFA for 1.5 h at 121 °C, and the products were reduced with $NaBH_4$ or $NaBD_4$ followed by acetylation. The resulting partially methylated alditol acetates were analyzed by GC and GC–mass spectrometry (GC–MS) using an SP-2380 capillary column (Supelco). The carrier gas was He (0.9 mL/min in GC and 0.5 mL/min in GC-MS), and the temperature programs were 60 °C for 1 min, 60 °C → 150 °C (30 °C/min), 150 °C → 180 °C (1 °C/min), 180 °C → 231 °C (30 °C/min), and 250 °C for 10 min in the split injection mode. The fragment ions and relative retention times were identified, and their molar percentages were estimated from the peaks and response factors [27].

2.5. Antimetastatic effect of GTE-II from the leaves of green tea on macrophages and NK cells

2.5.1. Animals and cell cultures

Specific pathogen-free (SPF), 6-week-old female BALB/c mice were purchased from Saeronbio Inc. (Gyeonggi, Korea). The mice were maintained in a clean rack in the SPF room of Department of Food Science and Biotechnology at Kyonggi University. Water and